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The intracellular domain of Notch ligand Delta1 induces cell growth arrest

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The article is dedicated to the memory of Tom Maciag, scientist, friend, and mentor.

Abstract Notch signaling involves proteolytic cleavage of the transmembrane Notch receptor after binding to its transmembrane ligands, Delta or Jagged; and the resultant soluble intracellular domain of Notch stimulates a cascade of transcriptional events. The Delta1 ligand also undergoes proteolytic cleavage upon Notch binding, resulting in the production of a free intracellular domain. We demonstrate that the expression of the intracellular domain of Delta1 results in a non-proliferating senescent-like cell phenotype which is dependent on the expression of the cell cycle inhibitor, p21, and is abolished by co-expression of constitutively active Notch1. These data suggest a new intracellular role for Delta1.

Keywords: Notch; Delta intracellular domain; p21; DNA synthesis; Senescence

1. Introduction

The Notch signaling pathway plays a critical role in cell fate determination at all stages of organism development [1]. The current model of the Notch signaling pathway suggests that the Notch transmembrane receptor molecule is activated via direct interaction with transmembrane ligands expressed on the surface of neighboring cells. This interaction results in consecutive cleavages of Notch by an ADAM metalloprotease and by a presenilin-dependent γ-secretase. The generated Notch intracellular domain (icd) translocates into the nucleus where it interacts with the transcription factors of the CSL family or activates CSL-independent signaling pathway(s) (for review see [2]).

We have demonstrated that the expression of the soluble extracellular domain of Delta1 (sDl1) enhances cell proliferation, and induces the non-classical release of FGF1 [3]. Recently Notch-dependent proteolytic cleavage was reported for *Drosophila* and mammalian Notch ligands of the Delta family [4–6]. Upon interaction with Notch, Delta is cleaved by an ADAM metalloprotease and presenilin/γ-secretase that release Delta icd from the plasma membrane. Immunohistochemistry experiments demonstrate that *Drosophila* Delta icd is able to enter the nucleus [5]. Interestingly, the icd of mammalian Delta1 (Dl1icd) contains a PDZ-binding site [7,8].

The ability of Delta to undergo proteolytic cleavage of its icd suggests a bidirectional character of Notch signaling. In the present study, we investigated the biological effects of Dl1icd expression in cell cultures. We demonstrate that Dl1icd induced p21-dependent blockage of DNA synthesis and cell proliferation arrest. Interestingly, constitutively active Notch1 (N1icd) was able to reverse Dl1icd1- induced phenotype.

2. Materials and methods

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVEC) (ATCC) at passages 7–12 were grown in EBM medium supplemented with EGM-2 growth factor cocktail (Cambrex). NIH 3T3 murine fibroblasts (ATCC), HEK293 cells (ATCC), p21−/−, p27−/− and wt mouse embryo fibroblasts (MEF) (gift of Dr. C. Sherr, St. Jude Children's Research Hospital in Memphis, Tennessee) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (Hyclone).

2.2. DNA constructs, transfection, preparation of adenoviruses and adenoviral transduction

To study the biological role of human Dl1icd, nucleotide sequence coding for amino acids 569–723 was cloned in pcDNA 3.1-Zeo vector (Invitrogen) in restriction sites *Xba*I and *Hin*dIII. Additionally,

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Abbreviations: CSL family, CBF, SuH, Lag-1; cdk, cyclin-dependent kinase; flDl1, full length Delta 1; HUVEC, human umbilical vein endothelial cells; Dl1icd, intracellular domain of Delta1; MEF, mouse embryo fibroblast; N1icd, Notch1 intracellular domain; NLS, nuclear localization sequence; sDl1, soluble Delta 1

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the V5 tag was introduced in the N-terminus of Dl1icd. NIH 3T3 cells were transfected using FuGene (Roche) transfection reagent according to the manufacturer's instructions. Selection of stably transfected NIH 3T3 cells was described earlier [9]. Dl1icd was also cloned in the multiple cloning site of the pAdlox shuttle vector (Invitrogen). The corresponding adenoviruses were prepared as described [10], and used to transduce HUVEC and MEF. In a series of experiments, an adenoviral construct expressing human N1icd [10] was used to transduce HUVEC 16–24 h before Dl1icd transduction. The control LacZ adenoviral construct was described earlier [10]. Alternatively, the control pcDNA3.1(−) Myc-His/LacZ construct (Invitrogen) was used for transient transfection. Full length human Delta 1 (flDl1) used for transient transfection was cloned into the EcoR1 and Bam H1 sites of the plasmid pcDNA3.1A (−) Myc-His (Invitrogen) [11]. The efficiency of Dl1icd and N1icd transduction was controlled using immunofluorescence anti-V5 staining, and it was always above 90%.

2.3. Site-directed mutagenesis

To mutate the nuclear localization sequences (NLS) of Dl1icd, we used a PCR-based strategy. Mutations were introduced with following primers: Dl1icd-nls1 – (s) cagaagcacgccccagccgacccctg and ggacggctggggcgtgcttctgcagcc (as); Dl1icd-nls2 – (s) gaagcatctgaacaaaggccggactcgggctgttc and (as) cagccgagtccggcctttgttcagatgcttctccaccc, using a Stratagene site-directed mutagenesis kit following the manufacturer's instructions. PDZ-binding site deletion mutant was generated by introducing a stop codon at amino acid 720 by using the following primers: (s) gatgagtgcgtctgagcaactgaggtftaa and (as) cacctcagttgctcagacgcactcatccttctc.

2.4. Immunoblot analysis

Lysates of LacZ- and Dllicd-transduced HUVEC were prepared, resolved by 12% or 15% SDS–PAGE and immunoblotted as described previously [12] using either a mouse anti-p21 (BD Biosciences), mouse anti-p27 (BD Biosciences), rabbit anti-cyclin A (Santa Cruz), rabbit anti-cyclin E (BD Biosciences), rabbit anti-cyclin D1 (Santa Cruz), rabbit anti-β-actin (Sigma) or rabbit anti-pErk1/2 antibodies (Cell Signaling).

2.5. Immunofluorescence confocal microscopy

Cells growing on glass coverslips were fixed 24 h after Dl1icd of flDl1 transfection with 4% (w/v) paraformaldehyde. Anti-V5 (Invitrogen) or anti-Myc (Covance) antibodies followed by FITC-conjugated secondary antibody were used to visualize, respectively, Dl1icd or flDl1. TO-PRO3 (Molecular Probes) was used to stain DNA as described previously [13]. Immunofluorescently stained cells were analyzed using a TC-SP confocal microscope (Leica).

2.6. DNA synthesis assay

[^{3}H]-Thymidine autoradiography was used to evaluate the levels of DNA synthesis as described previously [13]. The percentage of ^{3}H-labeled nuclei was calculated using an inverted Olympus microscope. In experiments with transient transfection, transfected cells were identified by immunoperoxidase staining as described [14] using antibodies against V5 or against β-galactosidase.

2.7. Acidic β-galactosidase staining

Cells transduced with Dl1icd were washed in PBS, fixed for 5 min in 2% formaldehyde/0.2% glutaraldehyde, washed, and stained for acidic β-galactosidase as described [15].

2.8. Real time RT-PCR

Total RNA from LacZ- and Dl1icd-transduced HUVEC was isolated using RNAeasy (Qiagen) according to the manufacturer's protocol. cDNA was obtained from 5 μg of total RNA with SuperScript™ (Invitrogen) reverse transcriptase by using an oligo(dT) primer (Invitrogen). Real-time PCR was performed using the Icycler IQ Real-Time PCR (Bio-Rad) according to the manufacturer's recommendations. Amplification of the *gapdh* cDNA was used as the endogenous normalization standard. Each sample was amplified in triplicate. The following specific primers were used for RT-PCR analysis of p21: (s) gattagcagcggaacaagga, (as) caactactcccagccccata.

3. Results

To study the biological effects of Dl1icd in cell culture, we transfected NIH 3T3 cells for further selection of cells stably expressing Dl1icd. Surprisingly, unlike sDl1 transfectants [3], cells transfected with Dl1icd failed to form colonies. Instead, Dl1icd transfectants surviving selection assumed morphology reminiscent of senescent fibroblasts: large, well-spread cells with hypertrophic cytoplasm (Fig. 1A). Since clones of stable Dl1icd transfectants did not arise, we prepared an adenoviral construct for Dl1icd expression, which allowed us to efficiently express Dl1icd in non-immortalized cells, such as HUVEC. To assess the ability of Dl1icd to inhibit DNA synthesis in HUVEC, Dl1icd- and control LacZ-transduced HUVEC were labeled with [^{3}H]-thymidine 48 h after transduction for a period of 16 h. The expression of Dl1icd resulted in the dramatic inhibition of DNA synthesis (Fig. 1B). Similar results were obtained with NIH 3T3 (data not shown) or HEK293 cell transduced with Dl1icd (Fig. 1F).

To further evaluate the status of Dl1icd-transduced HUVEC, we assessed the expression of β-galactosidase active at pH 6, a common biomarker of senescent non-immortalized cells [15]. Dl1icd transduction induced the activity of acidic β-galactosidase in HUVEC after 2 days, and most of the cells were acidic β-galactosidase positive after 4 days (Fig. 1C). The growth of Dl1icd-transduced HUVEC stopped, and cells remained viable and non-proliferating for at least 2 months (data not shown). Since cell senescence in vitro is normally accompanied by the reduction of telomere length [16], we assessed this parameter by using the Telomere Length Assay kit (Roche). Interestingly, no significant difference in telomere length was observed between Dl1icd- and LacZ-transduced cells 4 days after transduction (data not shown).

Recent studies demonstrated nuclear localization of *Drosophila* Delta icd [5]. To evaluate the ability of mammalian Dl1icd to localize into the nucleus, we transiently transfected HEK293 cells with C-terminally Myc-tagged human flDl1 and N-terminally V5-tagged Dl1icd. Confocal microscopy analysis, using the anti-Myc antibody, demonstrated cytoplasmic distribution of flDl1 (Fig. 1E). Conversely, Dl1icd was found both in the nuclei and cytoplasm of transfected cells (Fig. 1E). Analysis of the amino acid sequence of Dl1icd reveals two potential NLS domains – ^{575}KHRPP^{579} and ^{689}RKRPP^{692} (Fig. 1D). To investigate the functionality of Dl1icd NLS and their importance for Dl1icd biological effect, we prepared a series of mutants: in Dl1icd-nls1, amino acids ^{575}KHRPP^{579} were mutated to KHAP; and in Dl1icd-nls2, amino acids ^{689}RKRPP^{692} were mutated to RQP. In Dl1icd-nlsDM (double mutant), both hypothetical NLS in Delta1 were mutated as described above. While both Dl1icd-nls1 and Dl1icd-nls2 exhibited nuclear and cytoplasmic localization similarly to wild type Dl1icd, Dl1icd-nlsDM was detected exclusively in the cytoplasm of transfected cells (Fig. 1E). The autoradiographic studies of DNA synthesis in transiently transfected HEK293 cells demonstrated that when one or both of the Delta's NLS were mutated, the percentage of labeled nuclei was similar to that in the cells transduced with wild type Dl1icd, i.e., 3 times lower than in cells transfected with LacZ (Fig. 1F). In a similar series of experiments, we assessed the role of the C-terminal PDZ-binding domain of Dl1icd in its antiproliferative effect. We produced a deletion mutant of

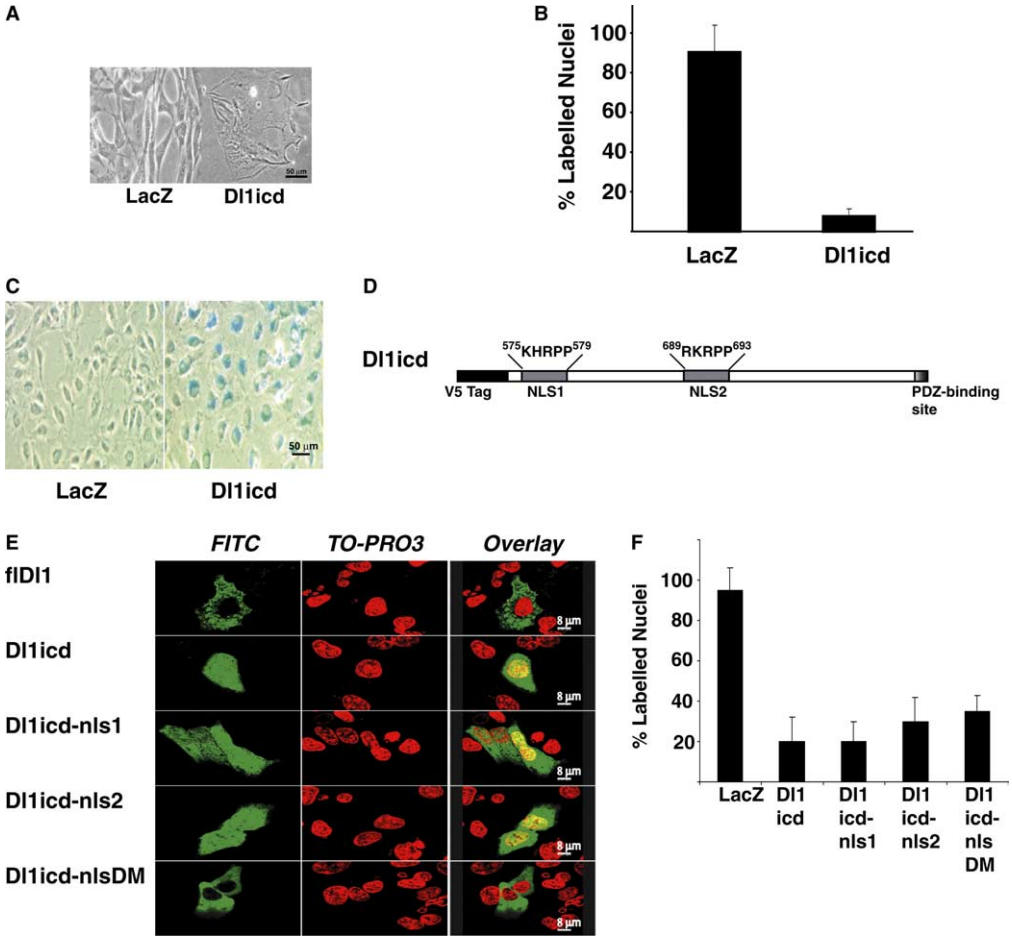

Fig. 1. Cells expressing Dl1icd adopt a senescent-like phenotype. (A) Cell morphology. Dl1icd-transfected NIH 3T3 cells 4 days after transfection and zeocin selection (phase contrast). (B) DNA synthesis. HUVEC were labeled for 16 h with 1 μCi/ml [^{3}H]-thymidine starting at 48 h after transduction with Dl1icd or LacZ. Bars represent average percentage of ^{3}H-labeled nuclei in LacZ- and Dl1icd-transduced HUVEC ± standard deviation (S.D.). (C) Acidic β-galactosidase expression. HUVEC adenovirally transduced with Dl1icd stained for acidic β-galactosidase 4 days after transduction. (D) Scheme of Dl1icd structure showing NLS and the PDZ-binding domain. (E) Nuclear localization of Dl1icd and its NLS mutants. HEK293 cells were transiently transfected either with flDl1, Dl1icd, or its corresponding NLS mutants as indicated. Cells were fixed, immunostained with anti-V5 antibody (Dl1icd and derived NLS mutants), or anti-Myc antibody (flDl1), and co-stained with TO-PRO3 48 h after transfection, and studied using confocal microscopy as described earlier [13]. (F) DNA synthesis. HEK293 were transiently transfected with LacZ, Dl1icd or Dl1icd-NLS mutants. DNA synthesis after transfection was determined using [^{3}H]-thymidine incorporation as described in Section 2. The average percentage of labeled nuclei ± S.D. is represented.

Dl1icd lacking the C-terminal PDZ-binding domain (^{721}TEV^{723}), and found that it induces the inhibition of DNA synthesis similarly to the wild type Dl1icd (data not shown). Thus, nuclear localization is not required for the anti-proliferative activity of Dl1icd, and PDZ-binding domain is dispensable for this effect. It can be hypothesized that PDZ-binding site is instead relevant to the interaction of transmembrane flDl1 with its cytoplasmic partners. The particular region(s) of Dl1icd required for the inhibition of cell proliferation remains to be elucidated.

Progression through the cell cycle is controlled by a group of cyclin-dependent kinases (cdks) and their inhibitory proteins [17]. Therefore, we assayed the expression of the cdk inhibitors, p21 and p27, in Dl1icd expressing cells. Western blot analysis revealed significant induction of p21 and p27 expression in HUVEC transduced with Dl1icd (Fig. 2A). At the same time, the expression of cyclins D1, A, and E as well as the levels of phosphorylated Erk 1 and Erk 2 in Dl1icd-transduced cells were not significantly changed (data not shown). We hypothesized that Dl1icd may induce growth arrest through upregulation of p21 or p27 expression or both. To assess this hypothesis, we utilized p21−/− and p27−/− MEF. Similarly to wild type (wt) MEF, Dl1icd transduction resulted in DNA replication blockage in p27 knockout MEF but the p21 knockout MEF were refractory to the inhibitory effect of Dl1icd (Fig. 2B). To further elucidate the stage of expression at which Dl1icd regulates p21 levels, we performed quantitative RT-PCR analysis and p21 promoter assay. We demonstrated that Dl1icd expression resulted in a strong increase of both p21 promoter activity in HEK293 cells (data not shown) and p21 mRNA levels in HUVEC (Fig. 2C).

Notch signaling determines the fate of many cell types through regulation of cell proliferation, differentiation, and apoptosis [1]. Since the expression of Notch1 overlaps the expression patterns of its ligands, Delta1 and Jagged1 [18–20], and since ligand-activated Notch cleavage results in the production of the soluble intracellular fragment of Notch, we sought to determine whether N1icd interferes with the biological effects of Dl1icd. To this end, we transduced HUVEC with N1icd-adenovirus 16 h prior to Dl1icd adenoviral transduction. Expression

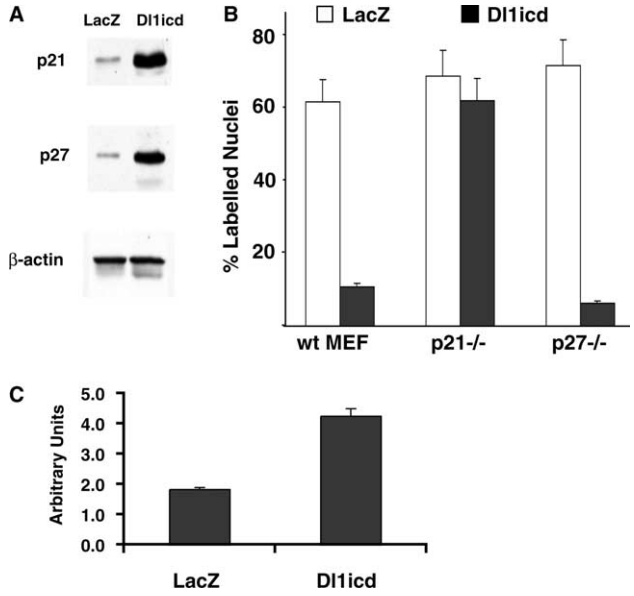

Fig. 2. Dl1icd-induced proliferation blockage is p21-dependent. (A) Dl1icd- and LacZ-transduced HUVEC were harvested 48 h after adenoviral transduction. Cell lysates were resolved by 15% SDS–PAGE and immunoblotted for cdk inhibitors, p21 and p27. Immunoblot for β-actin served as control of equal protein loading. (B) DNA synthesis in p21 and p27 knockout MEF expressing Dl1icd. p21−/−, p27−/−, and control wt MEF were labeled for 16 h with 1 μCi/ml [^{3}H]-thymidine starting at 48 h after transduction with Dl1icd. Bars represent average percentage of ^{3}H-labeled nuclei ± S.D. (C) Dl1icd induced expression of p21 mRNA in HUVEC. The expression of p21 was assessed by real time RT-PCR using the primers and conditions described in Section 2. The bars represent p21 mRNA levels normalized to *gapdh* mRNA levels ± S.D.

of N1icd abrogated the Dl1icd-induced senescence-like phenotype, as it was manifested by prevention of the expression of acidic β-galactosidase and of DNA synthesis blockage (Fig. 3B and A). Also, N1icd expression abrogated the induction of p21 expression by Dl1icd (Fig. 3C).

4. Discussion

We found that Dl1icd induced a p21-dependent inhibition of cell proliferation. Under the same experimental conditions the artificial expression of both LacZ and N1icd failed to inhibit DNA synthesis and, moreover, N1icd specifically abrogated the effect of Dl1icd, demonstrating that the antiproliferative activity of Dl1icd was not due to its overexpression. The irrelevance of nuclear localization of Dl1icd for its anti-proliferative effect indicated that Dl1icd does not participate directly in the activation of p21 transcription. This effect is most probably mediated through a cytoplasmic signaling pathway. Interestingly, HUVEC express Notch1, Delta1, and glycosyltransferase Lunatic Fringe (LFng) (data not shown). LFng potentiates the interaction between Notch1 and Delta1 [21]. The ability of HUVEC to proliferate may be maintained due to the simultaneous production of N1icd and Dl1icd, which may be a result of the efficient interaction of Notch 1 and Delta 1 promoted by LFng activity.

In the developing *Drosophila* wing, activation of Notch results in direct upregulation of cell proliferation without affecting cell fate determination [22]. In hematopoietic cells, the expression of N1icd results in delays of cell differentiation and diminishes the number of cells in the G_0/G_1 phase of the cell cycle, which also suggests induction of cell proliferation [23]. In order for proper Notch signaling to occur, there must be a distinction between a signaling cell versus a receiving cell. Based on the observations that Dl1icd induced non-proliferating phenotype, we suggest that its role in developing organisms is related to cell synchronization, tissue sculpting, and repair. In this scenario, at least three hypothetical situations may exist: (i) when a cell expressing Delta and Notch is surrounded by

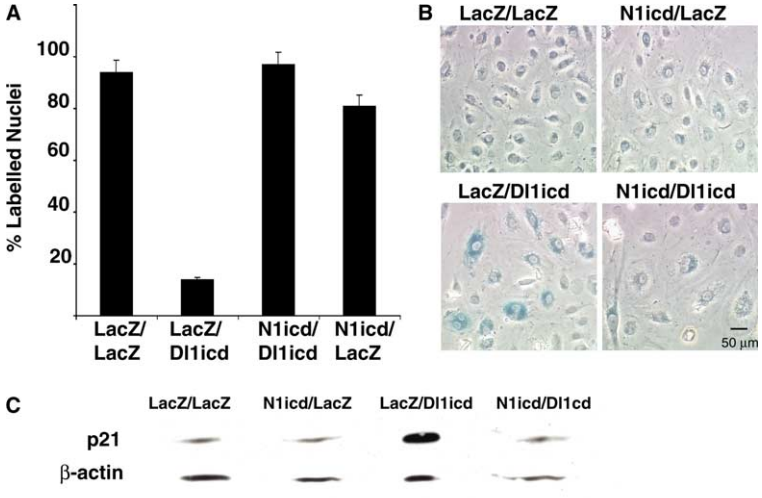

Fig. 3. N1icd expression prevents the effects of Dl1icd. (A) Dl1icd-induced inhibition of DNA synthesis. HUVEC were transduced with N1icd or LacZ, and 16 h later, the second transduction with Dl1icd or LacZ was performed. Cells were labeled for 16 h with [^{3}H]-thymidine 36 h after the second transduction [13]. The average percentages of [^{3}H]-thymidine-labeled nuclei ± S.D. are presented. (B) Dl1icd-induced acidic β-galactosidase activity. HUVEC were transduced with N1icd or LacZ; and 16 h later, cells were additionally transduced with either Dl1icd or LacZ. Cells were stained for acidic β-galactosidase 4 days after the second transduction. (C) p21 expression. HUVEC were transduced with N1icd or LacZ; and 16 h later, cells were additionally transduced with either Dl1icd or LacZ. 48 h later cell lysates were prepared, resolved by 15% SDS–PAGE, and immunoblotted for p21. Immunoblot for β-actin served as control of equal protein loading.

similar cells, the signals conducted through ligand and receptor are balanced and normal tissue homeostasis is maintained; (ii) when signaling through Notch is downregulated, e.g., by Numb [24], Delta signaling dominates over Notch signaling and cells stop proliferating as a result of Delta icd production; (iii) when Notch activation by Delta from a neighboring cell is potentiated by Fringe, the balance of signaling through Notch and Delta is skewed, and cell proliferation is upregulated.

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References

[1] Artavanis-Tsakonas, S., Rand, M.D. and Lake, R.J. (1999) Notch signaling: cell fate control and signal integration in development. Science 284, 770–776.
[2] Radtke, F. and Raj, K. (2003) The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat. Rev. Cancer 3, 756–767.
[3] Trifonova, R., Small, D., Kacer, D., Kovalenko, D., Kolev, V., Mandinova, A., Soldi, R., Liaw, L., Prudovsky, I. and Maciag, T. (2004) The non-transmembrane form of Delta1, but not of Jagged1, induces normal migratory behavior accompanied by fibroblast growth factor receptor 1-dependent transformation. J. Biol. Chem. 279, 13285–13288.
[4] LaVoie, M.J. and Selkoe, D.J. (2003) The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. J. Biol. Chem. 278, 34427–34437.
[5] Bland, C.E., Kimberly, P. and Rand, M.D. (2003) Notch-induced proteolysis and nuclear localization of the Delta ligand. J. Biol. Chem. 278, 13607–13610.
[6] Six, E., Ndiaye, D., Laabi, Y., Brou, C., Gupta-Rossi, N., Israel, A. and Logeat, F. (2003) The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase. Proc. Natl. Acad. Sci. USA 100, 7638–7643.
[7] Pfister, S., Przemeck, G.K., Gerber, J.K., Beckers, J., Adamski, J. and Hrabe de Angelis, M. (2003) Interaction of the MAGUK family member Acvrinp1 and the cytoplasmic domain of the Notch ligand Delta1. J. Mol. Biol. 333, 229–235.
[8] Six, E.M., Ndiaye, D., Sauer, G., Laabi, Y., Athman, R., Cumano, A., Brou, C., Israel, A. and Logeat, F. (2004) The Notch ligand Delta1 recruits Dlg1 at cell–cell contacts and regulates cell migration. J. Biol. Chem. 279, 55818–55826.
[9] Wessendorf, J.H., Garfinkel, S., Zhan, X., Brown, S. and Maciag, T. (1993) Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor. J. Biol. Chem. 268, 22100–22104.
[10] Small, D., Kovalenko, D., Soldi, R., Mandinova, A., Kolev, V., Trifonova, R., Bagala, C., Kacer, D., Battelli, C. and Liaw, L. (2003) Notch activation suppresses fibroblast growth factor-dependent cellular transformation. J. Biol. Chem. 278, 16405–16413.
[11] Han, W., Ye, Q. and Moore, M.A. (2000) A soluble form of human Delta-like-1 inhibits differentiation of hematopoietic progenitor cells. Blood 95, 1616–1625.
[12] Small, D., Kovalenko, D., Kacer, D., Liaw, L., Landriscina, M., Di Serio, C., Prudovsky, I. and Maciag, T. (2001) Soluble Jagged 1 represses the function of its transmembrane form to induce the formation of the Src-dependent chord-like phenotype. J. Biol. Chem. 276, 32022–32030.
[13] Andreeva, V., Prudovsky, I. and Maciag, T. (2004) Stimulation of quiescent cells by individual polypeptide growth factors is limited to one cell cycle. Eur. J. Cell Biol., 327–335.
[14] Prudovsky, I.A. and Tsong, T.Y. (1991) Fusion of fibroblasts with differentiated and nondifferentiated leukemia cells resulting in blockage of DNA synthesis. Dev. Biol. 144, 232–239.
[15] Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M. and Campisi, J. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA 92, 9363–9367.
[16] Bodnar, A.G., Ouelllette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S. and Wright, W.E. (1998) Extension of life-span by introduction of telomerase into normal human cells. Science 279, 349–352.
[17] Miele, L. (2004) The biology of cyclins and cyclin-dependent protein kinases: an introduction. Methods Mol. Biol. 285, 3–21.
[18] Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M.E. (1995) Notch signaling. Science 268, 225–232.
[19] Kimble, J. and Simpson, P. (1997) The LIN-12/Notch signaling pathway and its regulation. Annu. Rev. Cell. Dev. Biol. 13, 333–361.
[20] Weinmaster, G. (1997) The ins and outs of notch signaling. Mol. Cell. Neurosci. 9, 91–102.
[21] Haines, N. and Irvine, K.D. (2003) Glycosylation regulates Notch signalling. Nat. Rev. Mol. Cell. Biol. 4, 786–797.
[22] Baonza, A. and Garcia-Bellido, A. (2000) Notch signaling directly controls cell proliferation in the *Drosophila* wing disc. Proc. Natl. Acad. Sci. USA 97, 2609–2614.
[23] Carlesso, N., Aster, J.C., Sklar, J. and Scadden, D.T. (1999) Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. Blood 93, 838–848.
[24] Pece, S., Serresi, M., Santolini, E., Capra, M., Hulleman, E., Galimberti, V., Zurrida, S., Maisonneuve, P., Viale, G. and Di Fiore, P.P. (2004) Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. J. Cell Biol. 167, 215–221.